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Early intervention with gene-modified mesenchymal stem cells overexpressing interleukin-4 enhances anti-inflammatory responses and functional recovery in experimental autoimmune demyelination

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Abbreviations: Ad, adipose; BM, bone marrow; CNS, central nervous system; EF-1α, elongation factor-1α; eGFP, enhanced green fluorescent protein; EAE, experimental autoimmune encephalomyelitis; FBS, fetal bovine serum; IL-4, interleukin-4; i.p., intraperitoneal; IRES, internal ribosomal entry site; MSC, mesenchymal stem/stromal cell; MS, multiple sclerosis; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; PBS, phosphate buffered saline; STAT, signal transducer and activation of transcription; SIN, self-inactivating; Th, T helper; Tregs, T regulatory cells

Mesenchymal stem/stromal cells (MSCs) can be isolated from most adult tissues and hold considerable promise for tissue regenerative therapies. Some of the potential advantages that MSCs have over other adult stem cell types include: (1) their relative ease of isolation, culture and expansion; (2) their immunomodulatory properties; (3) they can provide trophic support to injured tissues; (4) they can be transduced by retroviral vectors at a high efficiency; (5) they have an ability to home to sites of inflammation and injury. Collectively these characteristics suggest that MSCs are attractive vehicles for cell and gene therapy applications. In the current study, we investigated whether transplantation of human adipose-derived MSCs (Ad-MSCs) engineered to overexpress the anti-inflammatory cytokine interleukin (IL)-4 was efficacious in experimental autoimmune encephalomyelitis (EAE). Ad-MSCs transduced with a bicistronic lentiviral vector encoding mouse IL-4 and enhanced green fluorescent protein (Ad-IL4-MSCs) stably expressed, relatively high levels of both transgenes. Importantly the phenotypic and functional attributes of Ad-IL4-MSCs, such as the expression of homing molecules and differentiation capacity, was not altered by the transduction process. Notably, the early administration of Ad-IL4-MSCs in mice with EAE at the time of T-cell priming attenuated clinical disease. This protective effect was associated with a reduction in peripheral MOG-specific T-cell responses and a shift from a pro- to an anti-inflammatory cytokine response. These data suggest that the delivery of Ad-MSCs genetically engineered to express anti-inflammatory cytokines may provide a rational approach to promote immunomodulation and tissue protection in a number of inflammatory and degenerative diseases including multiple sclerosis.

Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) believed to be orchestrated by autoreactive T-cells, which initiate and coordinate an immune-mediated attack on the myelin sheath and axons.[1] Current treatments for MS predominantly target the immune component of the disease and have only been effective in a relatively small proportion (~30%) of patients. Furthermore, patients with progressive neurological deterioration, due to the accumulation of axonal injury and neuronal loss, benefit little from existing therapies. On the basis of studies conducted in experimental autoimmune encephalomyelitis (EAE), the predominant animal model for MS, transplantation of mesenchymal stem/stromal cells (MSCs) may provide a novel therapeutic approach that can dampen autoimmune responses and promote endogenous neural and glial cell repair.

Originally identified in bone marrow (BM) cell cultures, MSCs are a non-hematopoietic cell population that can be isolated from a variety of adult tissues, including adipose (Ad) and umbilical cord.[2] In addition to their mesodermal differentiation potential,

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MSCs have the capacity to enhance repair processes and modulate the effector function of cells from the innate and adaptive immune systems.[3] Based on these properties, extensive efficacy testing of MSCs in pre-clinical animal models of inflammatory and degenerative diseases such as EAE has been performed. Several modes of action underpin their remarkable therapeutic value, including their ability to induce T-cell anergy,[4] inhibit T helper (Th)17 cells via a CCL2 antagonist,[5] polarize Th2 responses[6] and enhance neuroprotective mechanisms via the paracrine secretion of growth factors and antioxidant molecules.[7,8] Notably, systemically administered MSCs of either mouse or human origin have been found at distal sites in secondary lymphoid tissues and injured tissue sites, including the CNS, demonstrating that MSCs are capable of homing to inflammatory sites.[4,6,9,10]

Cytokines play a fundamental role in the inflammatory process in CNS autoimmunity. For example, altering cytokine pathways in EAE, as demonstrated in transgenic or knockout mouse models, has been shown to have a dramatic effect on the course of the disease.[11] Several mature $CD4^+$ T-cell effector subsets have been identified, each characterized by distinct effector functions and specific cytokine profiles.[12] In contrast to the pathogenic role of Th1/Th17 cells, Th2 cells, which produce interleukin (IL)-4, have been shown to mediate disease remission in animal models of autoimmunity.[13] IL-4 is a pleiotropic cytokine involved in innate and adaptive immunity. Among its many functions, IL-4 can activate T-cell growth and promote the differentiation of naïve T helper cells (Th0 cells) into Th2 cells. IL-4 signals via a heterodimeric receptor, inducing the activation of Janus kinase 1 and 3 and signal transducer and activation of transcription (STAT)6 molecules. These signaling events ultimately lead to the upregulation of the Th2 master regulator, GATA binding protein-3, and the subsequent transcriptional activation of target Th2-associated genes.[14] This includes *il-4,* which functions in a positive feedback loop to enhance IL-4 production. Concomitantly, IL-4 inhibits Th1 and Th17 differentiation via the transcription factors c-Maf and Gfi-1.[15,16] In addition to promoting Th2 differentiation, IL-4 also exerts a number of other immune functions, such as the induction of immunoglobulin class switching in B-cells,[17] alternative activation of macrophages[18] and the stimulation of Treg proliferation and chemotaxis.[19,20]

Evidence for the anti-inflammatory role of IL-4 in EAE originates from early studies demonstrating increased IL-4 mRNA and protein levels during disease remission.[21,22] The importance of IL-4 in regulating disease severity was initially indicated in IL-4 deficient C57Bl/6 mice immunized with guinea pig spinal cord myelin, which develop a more severe form of clinical disease in comparison to wild-type mice.[23] Moreover, it has been demonstrated that CNS-derived IL-4 is required to regulate EAE by way of modulating the activation of microglia.[24] Exogenous administration of IL-4 has been found to be beneficial, as intraperitoneal (i.p.) injection of IL-4 after adoptive transfer of myelin basic protein (MBP)-reactive T-cells decreased the clinical severity of EAE through the induction of Th2 cells.[25] Similarly, oral MBP in combination with IL-4 enhanced MBP-induced EAE suppression via the generation of Th3 regulatory cells that secrete transforming growth factor-β.[26] In order to overcome the need to deliver multiple doses of recombinant protein, a general consequence of the short half-life of cytokines in vivo, several investigations have adopted a gene therapy approach to deliver relatively consistent amounts of IL-4. For example, intrathecal administration of a herpes simplex virus type-1 vector engineered to express IL-4 has been shown to downregulate the pro-inflammatory cytokine milieu via the alternate activation of microglia.[27] Furthermore, injection of an IL-4-producing adenoviral vector was also found to attenuate EAE severity via the recruitment of Tregs.[19]

As an adjunct to these approaches, ex vivo gene modification of MSCs, which aims to further enhance their tissue regenerative and immune modulatory capacities, represents a promising tool for the delivery of anti-inflammatory cytokines. MSCs can be readily transduced by retroviral vectors and have a capacity for stable transgene expression[28] while maintaining their homing potential and multi-lineage differentiation capacity.[29] Several studies have demonstrated the efficacy of transplanting transduced MSCs expressing Th2 cytokines in inflammatory conditions. For example, MSC-based delivery of the anti-inflammatory cytokine IL-10 has been successfully applied to models of lung ischemia-reperfusion injury,[30] graft vs. host disease[31] and collagen-induced arthritis.[32] In the current study, the potential of Ad-MSCs to deliver IL-4 in EAE was explored. We demonstrate that human Ad-MSCs can be genetically engineered to secrete supraphysiological concentrations of mouse IL-4 without adversely affecting their phenotypic and functional properties. Furthermore, Ad-IL4-MSCs transplanted during the priming phase of EAE were able to reduce the inflammatory immune response, skew the pro-inflammatory cytokine profile and suppress disease severity. These data indicate that gene modified Ad-MSCs may provide another tool in the armamentarium of cellular and gene therapy approaches being developed for the treatment of MS.

Results

Ad-MSCs are efficiently transduced and express high levels of transgenic IL-4. To explore the potential for Ad-MSC based delivery of IL-4 for the treatment of inflammatory diseases such as EAE, we constructed a bicistronic lentiviral vector expressing mouse IL-4 under the transcriptional control of the elongation factor-1α (EF-1α) promoter. This vector (LV-IL4) also encoded an enhanced green fluorescent protein (eGFP) cassette translated from an internal ribosomal entry site (IRES) sequence to enable the identification and quantification of transduced cells using fluorescence techniques (**Fig. 1A**). Unconcentrated viral supernatant was used to transduce human Ad-MSCs twice at a multiplicity of infection of 50 and cells were characterized accordingly. Fluorescence microscopy of transduced cells (Ad-IL4-MSCs) showed that greater than 90% of cells expressed eGFP (**Fig. 1B**). Fortifying this observation, quantification of the number of transduced cells by flow cytometry revealed that 94% of Ad-IL4-MSCs expressed relatively high levels of eGFP (**Fig. 1C**). Significantly, Ad-IL4-MSCs expanded in culture for a further four passages retained this level of expression (data not shown), indicating stable transgene expression over time. To

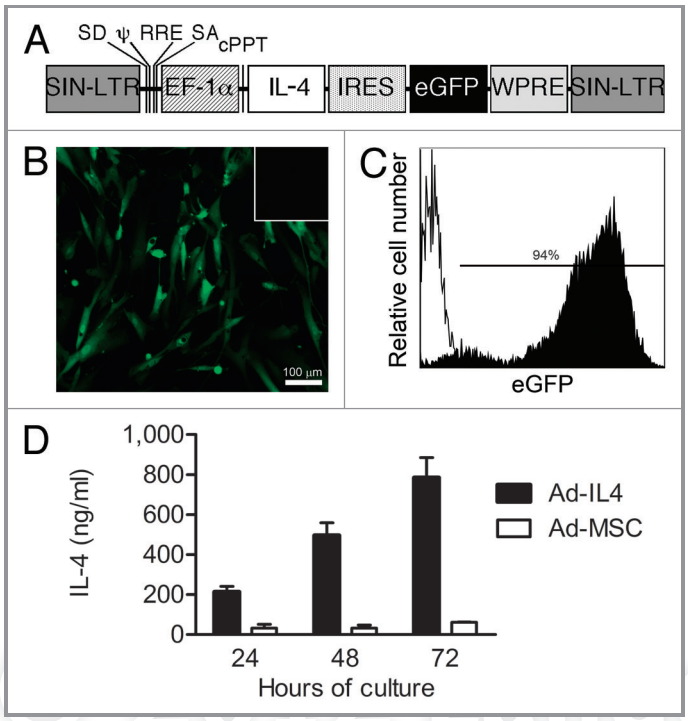

Figure 1. Schematic representation of recombinant lentiviral vector and in vitro expression of IL-4 in transduced Ad-MSCs. (A) The proviral, self-inactivating (SIN) form of LV-IL4 encoding mouse IL-4 under the transcriptional control of the EF-1α promoter. The vector incorporated an enhanced green fluorescent protein (eGFP) cassette under the translational control of an internal ribosomal entry site (IRES) sequence. LTR, long-term repeat; ψ, packaging signal; RRE, rev response element; SD and SA, splice donor and acceptor sites: WPRE, woodchuck hepatitis virus post-transcriptional regulatory element. (B) Expression of eGFP in Ad-IL4-MSCs and Ad-MSCs (insert) as determined by fluorescence microscopy. (C) Flow cytometric analysis of eGFP expression in Ad-IL4-MSCs (black histogram). Non-transduced Ad-MSCs (clear histogram) served as a negative control. Proportion of eGFP-positive Ad-IL4-MSCs is indicated. (D) Production of bioactive IL-4 in the supernatant of non-transduced (Ad-MSCs) and transduced (Ad-IL4-MSCs). Supernatant from cultured cells was retrieved on the indicated time points, processed and analyzed using a mouse-specific IL-4 ELISA assay. Results represent the mean + sem of two experiments each performed in duplicate with n = 2.

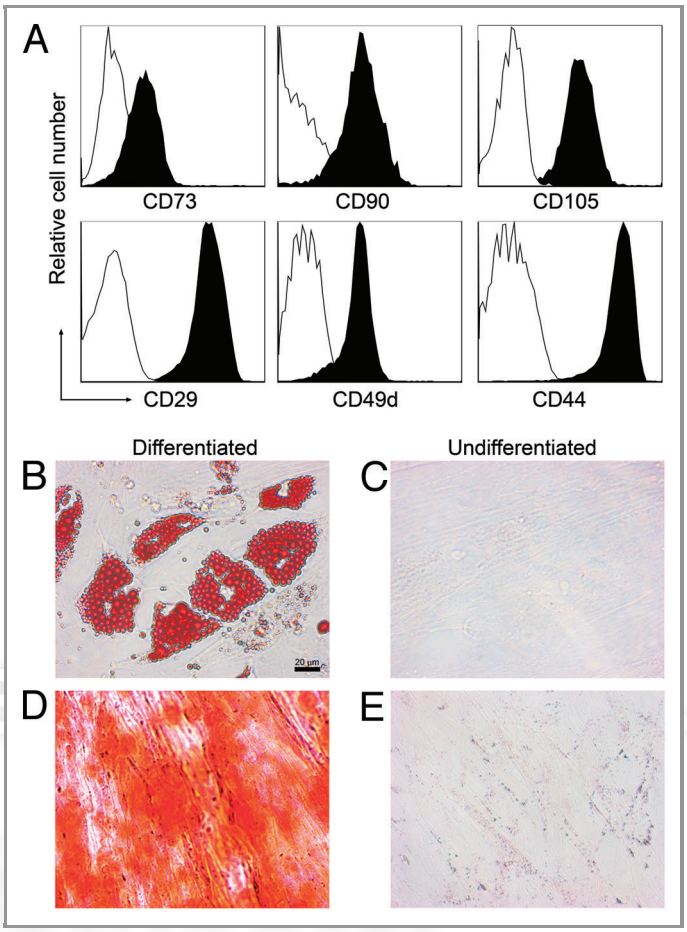

Figure 2. Phenotypic profile and differentiation potential of Ad-IL4-MSCs. (A) Single color flow cytometric analysis of Ad-IL4-MSCs stained with a panel of antibodies (black histograms) delineating antigens expressed on mesenchymal cells (CD73, CD90 and CD105) or adhesion molecules (CD29, CD49d and CD44). As a control, Ad-IL4-MSCs were stained with an isotype specific antibody (clear histograms) and analyzed accordingly. Histograms are representative of three experiments. Bright field images of Ad-IL4-MSCs cultures stained with Oil Red O (B and C) or Alizarin Red S (D and E) that were maintained in either differentiation medium (B and D) or basal medium (C and E). Oil Red O and Alizarin Red Staining demarcate cells belonging to the adipocyte and osteogenic cell lineages, respectively.

quantify the relative concentration of secreted IL-4, Ad-IL4-MSCs and non-transduced Ad-MSCs were established in culture and supernatant was collected after 24, 48 and 72 h. A linear increase in the production of IL-4 over time was observed from Ad-IL4-MSC supernatants, while IL-4 production from non-transduced Ad-MSC cultures was minimal (**Fig. 1D**).

Ad-IL4-MSCs maintain their phenotypic and functional properties. We sought to confirm whether Ad-IL4-MSCs retained their cell surface phenotype by assessing the expression of mesenchymal and hematopoietic markers by flow cytometry. Consistent with the minimal criteria described by Dominici et al.,[33] Ad-IL-4-MSCs expressed the canonical MSC markers CD73, CD90, CD105 as well as the cell migration molecules CD29 (integrin-β1), CD49d (integrin-α4) and CD44 (**Fig. 2A**).[34] Predictably, these cells lacked the expression of CD45, CD34, CD11b, CD19, HLA-DR and the co-stimulatory molecules CD80 and CD86 (data not shown). To determine whether transduced Ad-MSCs were still capable of mesodermal differentiation, a characteristic property of MSCs,[35] Ad-IL4-MSCs were cultured for two weeks in adipogenic differentiation medium. Phase contrast microscopy revealed that the cells displayed a morphology typical of adipocytes, which was confirmed by positive staining of lipid vacuoles with Oil Red O (**Fig. 2B**). Similarly, the osteogenic differentiation potential of Ad-IL4-MSCs after two weeks in osteogenic induction medium was verified by staining of calcium deposits with Alizarin Red S (**Fig. 2D**).

In addition to integrins and other adhesion molecules, MSCs have also been shown to express a range of chemokine receptors,[36,37] which are essential for homing to sites of inflammation. We therefore examined the mRNA expression of CCR1,

CCR2, CCR3, CCR5, CCR6, CCR7, CXCR3 and CXCR4 by RT-PCR, as these chemokine receptors are known to influence leukocyte trafficking in EAE.[38] Both transduced and non-transduced MSCs expressed mRNA for CCR1, CCR7 and CXCR3 as well as low but detectable levels for CCR3, CCR6 and CXCR4 (**Fig. 3**). Transcripts for the chemokine receptors CCR2 and CCR5 could not be detected in either cell population. Collectively these data suggest that the viral transduction process did not interfere with Ad-MSC differentiation programs, nor did it alter the cell surface phenotype and chemokine receptor transcript expression profile in these cells.

The immunosuppressive potential of transduced and non-transduced Ad-MSCs was initially compared using in vitro co-cultures incorporating limiting numbers of Ad-MSCs or Ad-IL4-MSCs in the presence of a fixed number of 2D2 splenocytes. T-cells from 2D2 mice, which transgenically express the Vα3.2 and Vβ11 T-cell receptor, specifically recognize the encephalitogenic myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide in the context of $I\text{-}A^b$.[39] Ad-IL4-MSCs and Ad-MSCs inhibited T-cell proliferative responses in a dose-dependent manner, with a significant reduction in T-cell proliferation observed at a ratio of 1:10 (**Fig. 4**). Notably, the suppressive effect exerted by Ad-IL4-MSCs was significantly greater than Ad-MSCs at this ratio. These data demonstrate that genetically engineered Ad-MSCs over-expressing IL-4 enhanced their immunosuppressive properties and reinforces the postulate that transplantation of this cell population may provide a novel approach for treating autoimmune demyelination.

Ad-IL4-MSCs delivered at the priming phase attenuate EAE severity. To examine the effects of Ad-IL4-MSC administration on the clinical course of EAE, we used a chronic progressive disease model induced by immunizing female C57Bl/6 mice with $MOG_{35–55}$. We first tested a treatment paradigm in which cells were administered on days 1, 3 and 5 post disease induction, in order to target T-cell priming within the lymph nodes and spleen. Ad-IL4-MSCs were administered i.p., as previous studies have shown that MSCs delivered by this route can attenuate peripheral immune responses and EAE severity.[5,40,41] Our results showed that the mean daily clinical score was reduced, although not significantly, in mice receiving Ad-MSCs compared with phosphate buffered saline (PBS)-injected controls. There was, however, a significant reduction in disease severity in mice receiving Ad-IL4-MSCs compared with PBS controls ($p < 0.01$, **Fig. 5A**). Interestingly, treatment with Ad-MSCs or Ad-IL4-MSCs reduced or prevented disease-related mortality, while a reduction in the maximum score in Ad-IL4-MSC-treated mice compared with PBS controls approached significance. No significant differences in other disease metrics, such as disease onset or cumulative score, were detected between the three treatment cohorts (**Table 1**). We next sought to determine whether Ad-IL4-MSCs delivered at the onset of clinical disease could influence the course of EAE. MSCs were administered on days 9, 10 and 11 (1×10^6 cells per injection) or days 12, 13 and 14 (5×10^6 cells per injection). In contrast to the earlier administration of cells, we did not observe any difference in the mean daily clinical score between cell-treated cohorts and controls

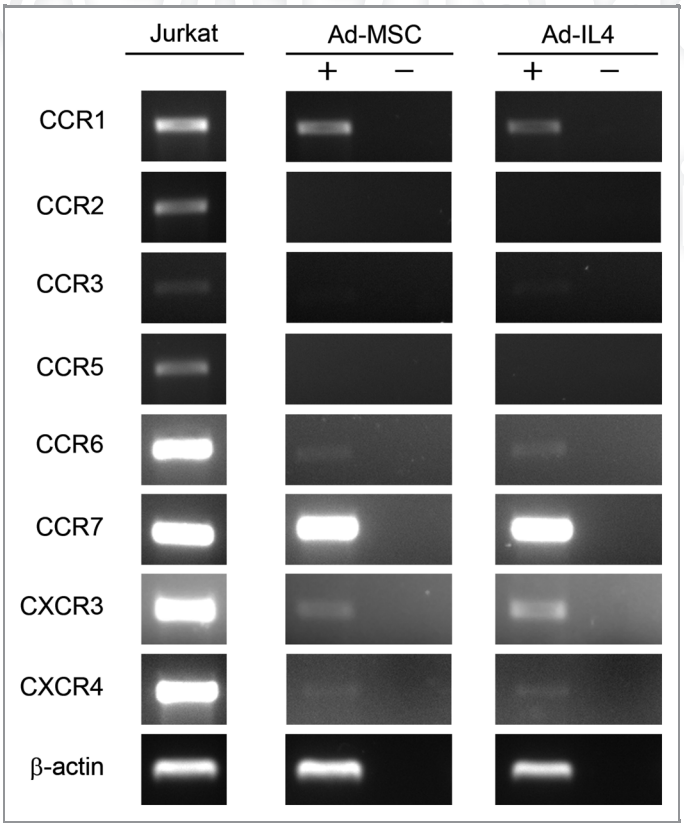

Figure 3. Gene expression profiles of non-transduced and transduced Ad-IL4-MSCs. Semi-quantitative RT-PCR analysis of human chemokine receptors transcripts in Jurkat cells (an immortalized human T-cell line known to express transcripts for all the surveyed genes), Ad-MSC and Ad-IL4 cells. As a positive control the housekeeping gene, β-actin, was amplified. To determine whether genomic DNA contamination occurred during RNA purification, reverse transcription of purified RNA was conduced with (+) and without (-) reverse transcriptase. The reverse transcribed cDNA was subsequently used as a template for PCR analysis.

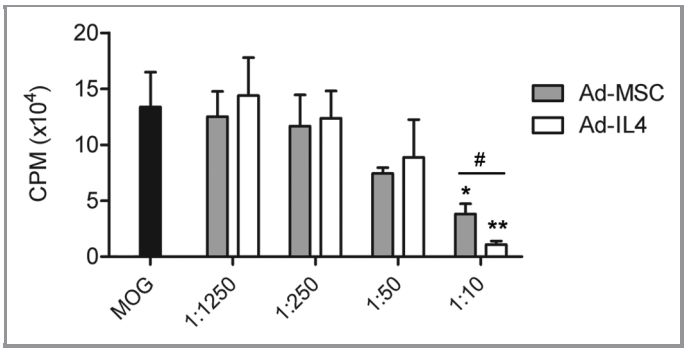

Figure 4. In vitro suppression of autoreactive T-cell proliferation. Splenocytes from 2D2 mice were stimulated with $MOG_{35–55}$ (black bar) in the presence of varying doses of Ad-MSCs (gray bars) or Ad-IL4-MSCs (white bars) (expressed as MSC:splenocyte ratio). Proliferative responses are expressed as the mean counts per minute (CPM) + sem of two independent experiments each performed in triplicate with n = 3 mice. *$p < 0.05$, **$p < 0.01$ vs. MOG-stimulated splenocytes without MSCs. #$p < 0.05$, Ad-MSC vs. AD-IL4-MSC.

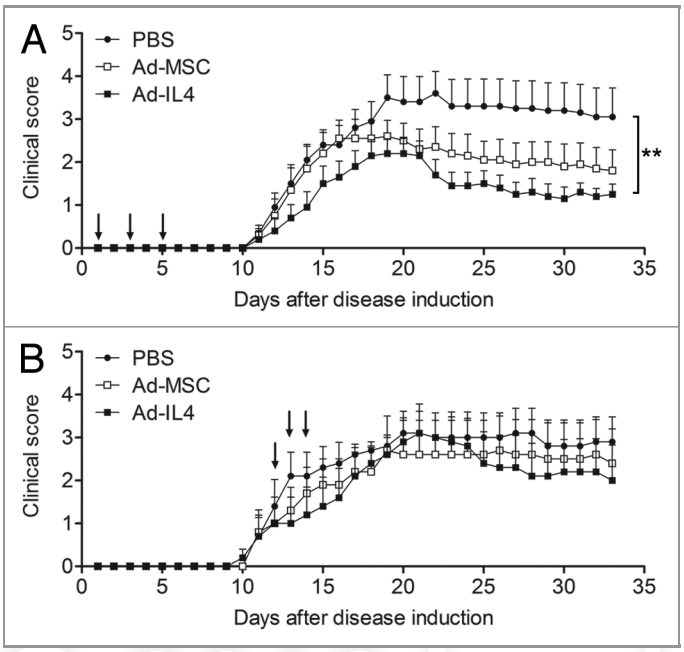

Figure 5. Transplantation of Ad-IL4-MSCs at the priming phase ameliorates EAE in C57Bl/6 mice. C57Bl/6 mice were immunized with MOG_{35-55} and injected with 1×10^6 Ad-MSCs, Ad-IL4-MSCs or PBS on days 1, 3 and 5 (A) or with 5×10^6 Ad-MSCs, Ad-IL4-MSCs or PBS on days 12, 13 and 14 (B) (as indicated by arrows). Data are expressed as the mean daily clinical score + sem with 5–10 mice per group. **$p < 0.01$ vs. PBS.

using either of these treatment paradigms (**Fig. 5B** and data not shown).

Ad-IL4-MSCs can promote Th2 responses. To determine whether early Ad-IL4-MSC treatment reduced EAE severity through modulation of peripheral immune cell responses, we analyzed T-cell proliferative recall responses to MOG_{35-55} or anti-CD3/28 from all animal groups. There was a trend toward a reduced proliferative response when splenocytes from Ad-IL4-MSC-treated mice were re-stimulated with MOG_{35-55} compared with PBS-injected mice, while there was a significant decrease in proliferation compared with the Ad-MSC-treated group ($p < 0.05$, **Fig. 6A**). Intriguingly, the T-cell proliferative response from anti-CD3/28 stimulated cultures was significantly higher in Ad-IL4-MSC-treated mice in comparison to Ad-MSC-treated animals (**Fig. 6B**).

We further hypothesized that the protective effect observed may have resulted from a skewing of the T-cell response from Th1/Th17 toward a Th2 phenotype. IL-4 can play an important role in directing the fate of naïve $CD4^+$ T-cells toward that of a Th2 phenotype, thereby inhibiting their differentiation into other effector T-cell lineages.[14] To explore this possibility, the cytokine secretion profile in supernatants from MOG_{35-55}-stimulated splenocyte cultures was examined. In splenocyte conditioned media from Ad-IL4-MSC-treated mice, the Th1 cytokine, IFNγ, was significantly reduced compared with mice receiving Ad-GFP-MSCs ($p < 0.05$, **Fig. 6C**). There was also a trend toward a decrease in production of IL-17A, a signature cytokine of Th17 cells (**Fig. 6E**), and a significant reduction in IL-6, a Th-17 inducing cytokine, compared with PBS controls ($p < 0.05$, **Fig. 6G**).[42] Although there was no significant difference in cytokine production between Ad-IL4-MSC-treated splenocytes and controls following anti-CD3/28 stimulation, there was an overall trend toward reduced secretion of pro-inflammatory cytokines (**Fig. 6D, F and H**). Together with IL-5 and IL-13, IL-4 is a signature effector cytokine secreted by Th2 cells. It was therefore interesting to note that the concentration of mouse IL-4 increased approximately 3-fold in splenocyte cultures from Ad-IL4-MSC-treated mice compared with Ad-MSC and PBS-treated animals (**Fig. 6J**), suggesting that Ad-IL4-MSCs promoted the differentiation of T-cells toward the Th2 lineage.

To determine whether Ad-IL4-MSC treatment led to higher amounts of circulating IL-4 in our pre-onset transplantation setting, we quantified the level of this cytokine in the sera of EAE mice 72 h after the last injection of cells (day 8). No difference in IL-4 levels was found between any of the three animal cohorts (**Fig. 7A**), suggesting that the beneficial effects exerted by Ad-IL4-MSCs were likely restricted to peripheral lymphoid tissues. To confirm whether Ad-IL4-MSCs were indeed acting in the peripheral lymphoid tissues of EAE mice to enhance Th2 type responses, we re-stimulated splenocytes from all three animal cohorts at the same time point (day 8) and quantified their production of IL-4. While IL-4 production by MOG_{35-55} stimulated splenocytes was similar between the three groups (**Fig. 7B**), splenocytes from Ad-IL4-MSC-treated mice produced significantly higher amounts of IL-4 upon stimulation with anti-CD3/28 (**Fig. 7C**). Collectively, these results suggest that early Ad-IL4-MSC treatment during the priming phase of disease has the potential to skew the peripheral T-cell response toward that of a Th2 phenotype, although this anti-inflammatory response was not sufficient to restrain Th1/Th17-mediated inflammatory responses and prevent neuroinflammation.

Discussion

In the current study, we investigated the immunoregulatory effect of gene modified MSCs overexpressing the anti-inflammatory cytokine IL-4 in chronic progressive EAE, a demyelinating animal model that reproduces some of the salient pathological hallmarks of MS. Our results demonstrate that when transplanted early in the disease

Table 1. Clinical features of EAE mice treated on days (d) 1, 3 and 5

Treatment	Disease incidence	Mortality	Disease onset (d)	Maximum score	Cumulative score
PBS	10/10	5/10	13.0 ± 0.7	3.8 ± 0.4	64.4 ± 10.7
Ad-MSC	10/10	1/10	13.7 ± 0.8	2.7 ± 0.4	45.8 ± 9.8
Ad-IL4-MSC	10/10	0/10	15.8 ± 1.8	2.4 ± 0.3	32.2 ± 5.5

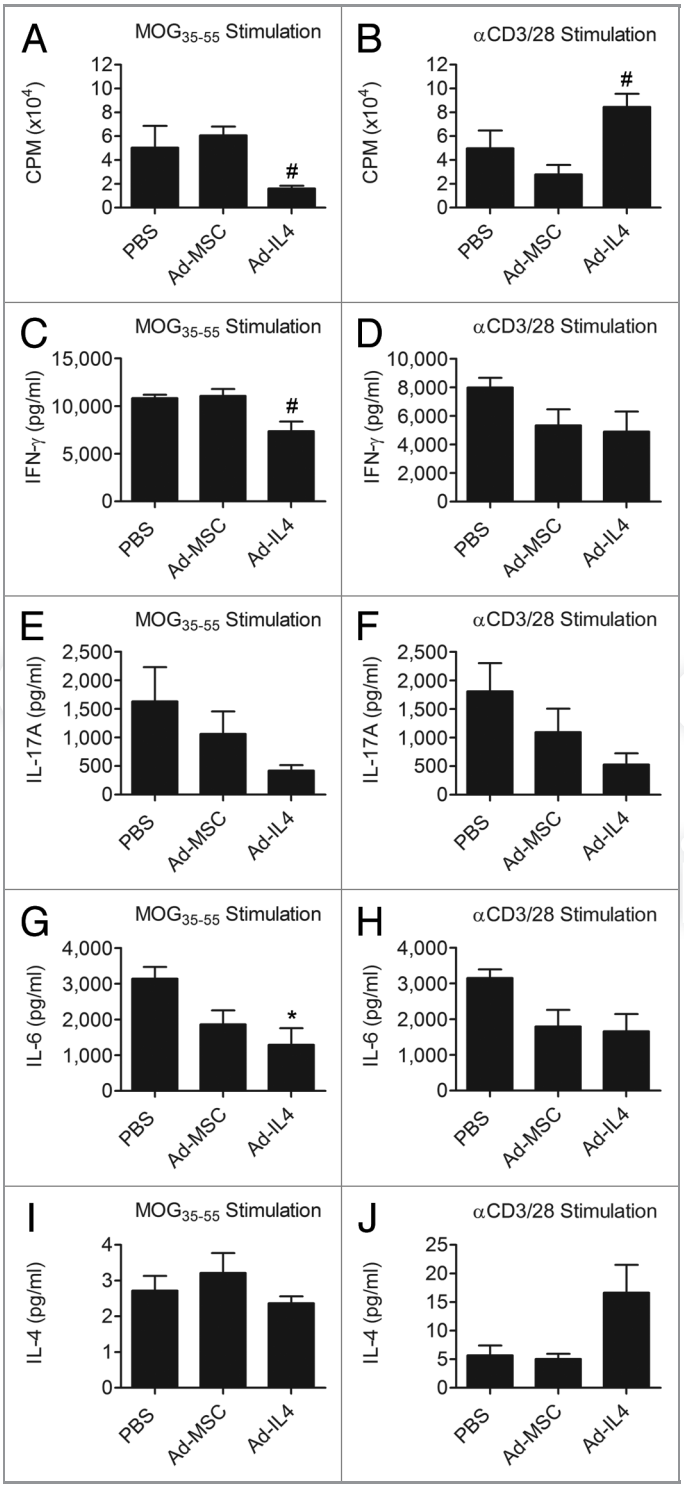

Figure 6. Transplantation of Ad-IL4-MSCs reduces pro-inflammatory T-cell responses. Splenocytes from EAE mice treated at the priming phase were isolated after 30 d and stimulated in vitro with MOG_{35-55} (B) or with anti-CD3/28 antibodies (C). Proliferative responses are expressed as the mean counts per minute (CPM) + sem of two independent experiments each performed in triplicate with n = 5 mice. In parallel cultures, supernatants from MOG_{35-55} (D, F and H, J) or anti-CD3/28 (E, G, I and K) stimulated splenocyte cultures were collected after 48 h and cytokine content analyzed by cytokine bead array. Data were generated from 5 to 10 animals per group that were pooled from two independent experiments and are expressed as the mean + sem. *$p < 0.01$ vs. PBS, #$p < 0.05$ vs. Ad-MSC.

course, delivery of gene modified Ad-MSCs resulted in improved clinical symptoms over their non-transduced cellular counterparts, by way of enhancing immune suppressor mechanisms.

Among the Th cell subsets identified to date, Th1 and Th17 cells have been identified as key purveyors of the autoimmune response in MS.[43] In contrast, Th2 cells, which are pathogenic in allergic inflammatory disease settings such as asthma,[44] exert a protective effect.[45,46] Studies dissecting the pathogenic role of T cells have clearly demonstrated that manipulating cytokines which control T-cell differentiation and effector function can dramatically alter the course of EAE.[47] One potential cytokine that has been investigated is IL-4. Systemic administration of IL-4 has been shown to ameliorate EAE induced by the adoptive transfer of MBP-specific T-cells via a Th1/Th2 shift.[25] A drawback to this approach is that the serum half-life of IL-4 is short (only 19 min in mice).[48] Indeed, injection of recombinant IL-4 every 8 h for a period of at least 6 d was required to produce a therapeutic effect in an adoptive transfer model of EAE.[25] To overcome this obstacle, several investigations have employed gene delivery systems for sustained maintenance and heightened expression of encoded proteins. In view of this, suppression of EAE has been demonstrated in animals transplanted with gene modified T-cells expressing IL-4,[49,50] or injected with recombinant plasmid,[51] adenoviral[19] and herpes simplex viral vectors[27,52,53] encoding IL-4. However, a number of limitations exist with these approaches, which include the invasiveness of vector administration, vector immunogenicity and lack of long-term expression of the therapeutic transgene. To overcome these obstacles, we sought to examine the potential of Ad-MSCs to act as a cellular vehicle to deliver IL-4, as MSCs are known to home to areas of tissue inflammation and possess broad immunoregulatory properties.[54] Consistent with this theme, several reports have demonstrated the immune modulating capabilities of transplanted human BM-MSC[6,8,10,41,55] and murine Ad-MSCs[56] in mice with EAE. We have also compared the efficacy of three MSC types isolated from the BM, umbilical cord and Ad tissue and have demonstrated that Ad-MSCs appear more efficacious at reducing EAE disease in comparison to the other cell types (Payne et al., unpublished data).

Characterization of transduced Ad-MSCs revealed that these cells were highly amenable to lentiviral-mediated transduction, as nearly 100% of cells expressed eGFP. We further demonstrated that Ad-IL4-MSCs were capable of expressing relatively high levels of eGFP, which correlated with the production of supraphysiological levels of IL-4 over time. Together these results are consistent with other reports demonstrating that transduction of MSCs is highly efficient[28,29] and thus suggest that Ad-MSCs may represent a viable cellular vehicle for the systemic delivery of therapeutic molecules.

Current minimal criteria used to define MSCs, which are an inherently heterogeneous population, incorporate the expression of a panel of surface markers as well as a demonstrable ability for

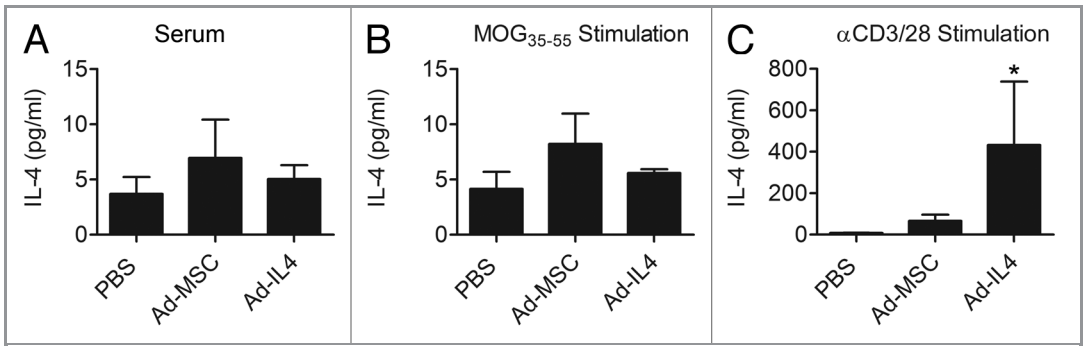

Figure 7. The beneficial effect of Ad-IL4-MSCs is restricted to peripheral lymphoid tissues. Sera and splenocytes from EAE mice treated at the priming phase were isolated after 8 d. The level of IL-4 in the serum (A), supernatants from MOG_{35-55} (B) or anti-CD3/28 stimulated splenocyte cultures (C) were quantified by cytokine bead array. Data are expressed as mean + sem with 5 mice per group. *$p < 0.01$ vs. PBS.

multipotent differentiation.[33] In vitro culture conditions can impact on several MSC attributes, such as their replicative senescence, multipotentiality, expression of homing molecules and possibly other functional properties relevant to their in vivo efficacy.[37,57,58] Given this, we performed a number of analyses on Ad-IL4-MSCs and found that the culture conditions and the transduction protocol employed did not impact on cell surface marker expression nor adversely affected their growth or differentiation potential. Of particular note, Ad-IL4-MSCs maintained expression of the adhesion molecules CD44, the VLA-4 heterodimers, CD49d/integrin-α4 and CD29/integrin-β1, and the chemokine receptors CCR1, CCR3, CCR6, CCR7, CXCR3 and CXCR4, which are important mediators of cell trafficking in EAE. In accordance with data showing that human BM-MSCs can migrate in response to CCL3, CCL5 and CXCL10,[59] the expression profile of adhesion molecules and chemokine receptors presented herein suggests that gene-modified Ad-MSCs have the capacity to home to inflammatory sites. Indeed, several investigations have shown that transplanted Ad-MSCs can migrate to a number of anatomical locations,[60] including areas of tissue injury within the kidney, CNS and lymphoid tissues.[56,61,62] We demonstrated that serum IL-4 did not differ between mice transplanted with Ad-IL4-MSC and controls; however, there was an increase in the production of IL-4 by splenocytes from Ad-IL4-MSC-injected mice, suggesting a shift toward a Th2 type immune response. These data support the notion that gene modified Ad-MSC have the capacity to migrate to peripheral lymphoid tissues and exert their immunoregulatory effects within these organs.

Administration of gene-modified Ad-MSCs during the priming phase of the disease reduced disease progression in C57Bl/6 mice immunized with MOG_{35-55}. The canonical cytokine pattern in EAE is generated predominantly from Th1 and Th17 pro-inflammatory cells. In our study, transplantation of Ad-IL4-MSCs suppressed T-cell proliferation in recall cultures stimulated with MOG_{35-55} and decreased the production of pro-inflammatory cytokines, including IFN-γ, IL-6 and IL-17. An intriguing observation was the enhanced proliferative response of anti-CD3/28 stimulated splenocytes from Ad-IL4-MSC-injected mice, which also displayed an increased capacity to produce IL-4. Various signaling cascade components such as kinases, phosphatases, adaptor molecules and molecular scaffolds are required to transmit extracellular signals via the T-cell receptor/CD3 complex to generate cellular responses.[63] In the context of our results, we hypothesize that in the presence of IL-4, T-cell stimulation with antigen-specific or non-specific mitogens can activate dichotomous signal transduction pathways leading to divergent outcomes.

While we have demonstrated that suppression of EAE was mediated in-part by the skewing of a pro-inflammatory Th1/Th17 response in favor of an anti-inflammatory, Th2-biased response, several investigations have revealed that IL-4 regulates many aspects of innate and adaptive immunity. For example, IL-4 can support naive Th0 precursors to develop into Th2 cells that secrete IL-4 and IL-5, but little IL-2 or IFN-γ[64] as well as promote the growth and migration of Tregs.[19,20] Notably, IL-4 has also been shown to induce the activation of anti-inflammatory macrophages in the periphery and in the CNS,[24] and inhibit the release of certain inflammatory mediators such as IL-1β, IL-8, TNFα and PGE2.[65] These reports suggest that IL-4 can target a number of pro-inflammatory molecules and alter the immunological response, favoring the development of an anti-inflammatory milieu. We have demonstrated that, in contrast to the early administration of Ad-IL4-MSCs, transfer of Ad-IL4-MSCs during disease onset did not ameliorate the signs of EAE. This result may be attributed to the minimal number of cells that could migrate to the CNS,[41] where expression of IL-4 is specifically required at that clinical stage to attenuate disease.[52] Alternatively, the pro-inflammatory environment coupled with ongoing demyelination and axonal loss, even at the onset stage of the disease,[66] may have overcome the suppressive and reparative mechanisms mediated by Ad-IL4-MSCs. In this context, it is noteworthy that the efficacy of current MS therapies is dependent on how soon treatment is initiated. The general consensus suggests that intervention must begin immediately after diagnosis, or at a time when the patient has partially or fully recovered from the initial episode of disease. While approved treatments have been shown to have some efficacy during the inflammatory disease stage, these have little or no effect once neurodegenerative processes predominate.[66]

In summary, we have shown that transplantation of gene modified Ad-MSCs can inhibit MOG-specific T-cells, which are

at the fulcrum of EAE progression in C57Bl/6 mice immunized with $MOG_{35–55}$. Furthermore, we described that a shift in the cytokine response from Th1/Th17 to Th2 was an underlying mechanism that suppressed CNS autoimmunity. While the lack of MHC class II and co-stimulatory molecules suggests that these cells are cloaked from the host immune system, several investigations have reported that MSCs can upregulate these molecules under inflammatory conditions, thus rendering these cells immunogenic.[67,68] Although the in vivo distribution and homing behavior of these cells in immunocompetent animals remains poorly characterized, the premature loss of donor cells under inflammatory conditions will undoubtedly impact their efficacy. Approaches that control host immune responses to prolong MSC grafts will need to be addressed. Despite these challenges, MSCs overexpressing anti-inflammatory molecules potentially offer a novel mode for the treatment of a variety of inflammatory and degenerative diseases.

Materials and Methods

MSC culture. Human Ad-MSCs, purchased from ScienCell (7510), were cultured in tissue culture-treated flasks pre-coated with 2 μg/cm^2 poly-L-lysine (Sigma-Aldrich, P4274) in α-minimal essential media (Invitrogen, 12561-056) supplemented with 5% fetal bovine serum (FBS, Invitrogen, 10099-141), 100 U/ml penicillin/100 μg/ml streptomycin (Invitrogen, 15140-122) and 1% MSC growth supplement (ScienCell, 7552). Cells were seeded at a density of 5,000 cells/cm^2 and passaged when they reached 90% confluence using 0.25% (w/v) trypsin-EDTA (Invitrogen, 25200-056).

Lentiviral transduction. The parental pWPI lentiviral plasmid, a second generation, self-inactivating, bicistronic lentiviral vector, was obtained from Professor Didier Trono (Ecole Polytechnique Federale de Lausanne). This vector backbone incorporates an EF-1α promoter, an IRES-eGFP cassette and the Woodchuck post translation regulatory element. Using standard molecular biology techniques, the cDNA for mouse IL-4 (Open Biosystems, MMM1013-7510313) was subcloned and blunt-end ligated upstream of the IRES-eGFP cassette generating the transfer vector pWPI-IL4-IRES-eGFP. Viral stocks were generated by transfecting pWPI-IL4-IRES-eGFP together with psPAX2 and pMD2.G using Fugene6 (Roche, 11988387001) into 293T cells (ATCC CRL-11268). Supernatants were collected, passed through a 0.22 μm filter and titers calculated after flow cytometric determination of eGFP in transduced HeLa cells (ATCC CCL-2). Typically, 0.5–1 × 10^7 transducing units/ml of unconcentrated vector was generated in this manner. Passage 2 Ad-MSCs were transduced twice at a multiplicity of infection of 50 over a 48 h period with unconcentrated lentiviral supernatant diluted 1:2 with culture medium in the presence of 8 μg/ml protamine sulfate (Sigma-Aldrich, P4020). Transduction medium was replaced with fresh medium 16 h after each transduction. Transduced cells were expanded and cryopreserved at passage 5 until required.

Quantification of secreted IL-4. Ad-IL4-MSCs and Ad-MSCs were seeded in 6-well tissue culture-treated plates at 5,000 cells/cm^2 and supernatant was collected after 24, 48 and 72 h. IL-4 production was quantified using a mouse IL-4 OptEIA ELISA kit (BD Bioscience, 555232) according to the manufacturer's instructions.

Mesodermal differentiation. Adipogenic and osteogenic differentiation assays were performed using a Human MSC Functional Identification Kit (R&D Systems, SC006) according to the manufacturer's instructions. To detect lipid vacuoles, which are characteristic of cells of the adipogenic lineage, cells were washed with D-PBS (Invitrogen, 14190), fixed for 1 h in 2 ml 10% neutral-buffered formalin (Sigma-Aldrich, HT501128), washed with PBS and then stained with 2 ml 0.3% (w/v) Oil Red O (Sigma-Aldrich, O0625) for 10 min at room temperature. To detect calcium deposits, which are characteristic of cells differentiating along the osteogenic lineage, cells were washed with D-PBS, fixed for 1 h in 2 ml 10% neutral-buffered formalin, washed in deionized water and then stained with 2 ml 1% (w/v) Alizarin Red (Sigma-Aldrich, A5533) for 10 min.

Cell surface phenotyping. Phenotypic analysis of Ad-IL4-MSCs was performed by staining 1 × 10^6 cells with 30 μl of optimally-diluted primary antibody on ice for 20 min. Cells were washed in FACS buffer [D-PBS containing 1% FBS, 5 mM EDTA, (BDH, 10093.5V), 0.02% (w/v) sodium azide (Sigma-Aldrich, S2002)] and acquired on a FACSCalibur flow cytometer (BD Biosciences). Data was analyzed using Gatelogic software (Inivai Technologies). All primary and isotype control antibodies are listed in **Table 2**.

EAE induction and cell transplantation. EAE was induced in young female C57Bl/6 mice aged 8 to 12 weeks as previously described.[69] Ad-IL4-MSCs or Ad-MSCs were administered i.p. at days 1, 3 and 5 (1 × 10^6), days 9, 10 and 11 (1 × 10^6) or days 12, 13 and 14 (5 × 10^6) post disease induction in a total volume of

Table 2. Antibodies reactive against human surface antigens

Antigen	Company	Catalog No.
CD11b	BD	555388
CD19	BD	555415
CD29	BD	559883
CD34	BD	555822
CD44	BD	560533
CD45	BD	555485
CD49d	BD	555503
CD73	BD	550257
CD80	BD	559370
CD86	BD	555660
CD90	BD	555597
CD105	eBioscience	17-1057-42
HLA-DR	BD	559866
Ms IgG1κ	BD	550617
Ms IgG1κ	BD	555751
Ms IgG1κ	BD	555750
Ms IgG2aκ	BD	550882
Ms IgG2bκ	BD	560542

200 µl. Control mice received an equal volume of PBS. Neurological signs were determined using an arbitrary clinical score as previously described.[70] All animal experiments were performed in accordance with the Australian code of practice for the care and use of animals for scientific purposes (2004, 7th edition), after approval by the Monash University School of Biomedical Sciences Animal Ethics Committee.

T-cell proliferation assays and cytokine production. Spleens were dissected and single cell suspensions prepared in complete medium [RPMI 1640 (Invitrogen, 21870-092) containing 10% heat-inactivated FBS, 2 mM L-glutamine (Invitrogen, 25030-081), 100 U/ml penicillin/100 µg/ml streptomycin, 50 µM 2-mercaptoethanol (Invitrogen, 21985-023) and 1 mM sodium pyruvate (Sigma-Aldrich, S8636)]. Following red blood cell lysis [1 min incubation with 155 mM NH_4Cl (Merck, 101145), 10 mM $KHCO_3$ (Merck, 104928), 0.1 mM EDTA (BDH, 10093.5V)] and 2.5×10^5 splenocytes were added in triplicate to 96-well, flat bottom microtiter plates (Nunc). Splenocytes were cultured in complete medium alone or in the presence of either 20 µg/ml $MOG_{35–55}$ or 800 ng/ml ionomycin (Sigma-Aldrich, I0634) and 20 pg/ml PMA (Sigma-Aldrich, P1585), or in wells pre-coated with 10 µg/ml anti-CD3 (BD Bioscience, 553057) and 10 µg/ml anti-CD28 antibodies (BD Bioscience, 553294) to a final volume of 200 µl. Cells were incubated at 37°C for 72 h with the addition of 1 µCi/well [^{3}H]-thymidine (Bioscientific, ART-0178D) for the last 18 h of culture. Cells were harvested onto filter mats (Perkin Elmer, 6005422) and incorporated radioactive nucleic acids counted using a Top Count NXT Scintillation Counter (Packard Biosciences). For co-culture experiments, Ad-MSCs or Ad-IL4-MSCs were added at concentrations ranging from 0.002 to 0.25×10^5 cells per well as per the above conditions, prior to the addition of 2.5×10^5 splenocytes.

For analysis of cytokine production, 2.5×10^6 splenocytes were cultured in 24-well plates for 48 h in complete medium alone, or media supplemented with 20 µg/ml $MOG_{35–55}$ or 10 µg/ml anti-CD3/anti-CD28, to a final volume of 1 ml. For analysis of serum IL-4, blood was collected by cardiac puncture and allowed to clot at room temperature for 2 h. Sera was collected, clarified by centrifugation and stored at -20°C until required. Quantitative analysis of IL-4, IL-6, IL-17A and IFNγ was performed using a mouse cytometric bead array kit (BD Bioscience, 560485) according to the manufacturer's instructions. Acquisition of events was performed on a FACSCanto II flow cytometer (BD Bioscience) and data analyzed and fitted to a 4-parameter logistic equation using the FCAP array software (Soft Flow).

Statistical analysis. Data are presented as the mean + the standard error of the mean (sem). All statistical analyses were performed using Prism 5.04 (Graphpad software). Significant differences between two groups were determined using an unpaired Student's t-test. Statistical analysis of three or more groups was performed using one-way ANOVA with Bonferroni post-test or Kruskal-Wallis with Dunn's post-hoc test for non-parametric data. p values of less than 0.05 were considered to be significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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